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On the measurement of the functional properties of the CFTR

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Abstract

A number of methods are currently employed to assess the functional properties of CFTR channels and their response to pharmacological potentiators, correction of the defective CFTR trafficking, and vectorial introduction of new proteins. Here we review the most common methods used to assess CFTR channel function. The suitability of each technique to various experimental conditions is discussed.

Keywords: CFTR; Chloride transport; Ionic current; Membrane potential; Chloride flux

1. Introduction

Cystic fibrosis (CF) is caused by a genetic defect in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein, the only known member of the ATP-Binding Cassette (ABC) family that behaves as an ion channel. In fact, when CFTR pore opens, the flux of anions across the cell membrane is driven only by their electrochemical potential [1]. As most members of the ABC family, CFTR is composed of a transmembrane domain (TMD), formed of 6 transmembrane segments, followed by an intracellular nucleotide binding domain (NBD). This motif is repeated twice, with the first NBD linked to the second TMD by a regulatory domain (RD), located in the intracellular compartment (see Fig. 1A), that contains a considerable number of putative phosphorylation sites [2].

CFTR is activated by protein kinase A (PKA), and probably also by protein kinase-C (PKC) [3] phosphorylation at multiple sites located at the RD. Because channel phosphorylation by PKA is mandatory for channel activity, CFTR channel is also defined as a "cAMP-activated channel". Phosphorylated CFTR channels are regulated by intracellular ATP. The most accepted model of CFTR gating proposes that binding of ATP promotes the dimerisation of the NBDs, leading to a conformational change at the level of the TM domains that in turn leads to channel opening (Fig. 1B). The hydrolysis of ATP by the enzymatic activity of the NBDs terminates the activity cycle, releasing ADP (see Fig. 1C). Interestingly, this activity cycle is not reversible (no ATP can be synthesised from ADP), but the energy liberated by the hydrolysis of ATP is not used to transport chloride. Indeed, once phosphorylated, ATP can be opened by non-hydrolysable ATP analogues, such as 5′-adenylyl-beta,gamma-imidodiphosphate (AMP-PNP) [4,5].

Immunological localisation of CFTR has revealed that it is abundant in epithelial cells, including those lining the sweat ducts, small pancreatic ducts, intestinal crypts, lung epithelia, and the epithelia of kidney tubules, where the protein appears to be restricted to the apical membrane. The cell and tissue distributions of the CFTR chloride channel are consistent with a function in vectorial ion movements across the epithelium. Several reports have indicated that CFTR is also expressed in the heart and in the central nervous system, but its functional relevance has not yet been elucidated.

Although the exact mechanisms leading to CF pathology is still a question of debate, the disease is associated to a strong reduction/absence of chloride ion transport across the apical membrane of the epithelium. There is a clear correlation

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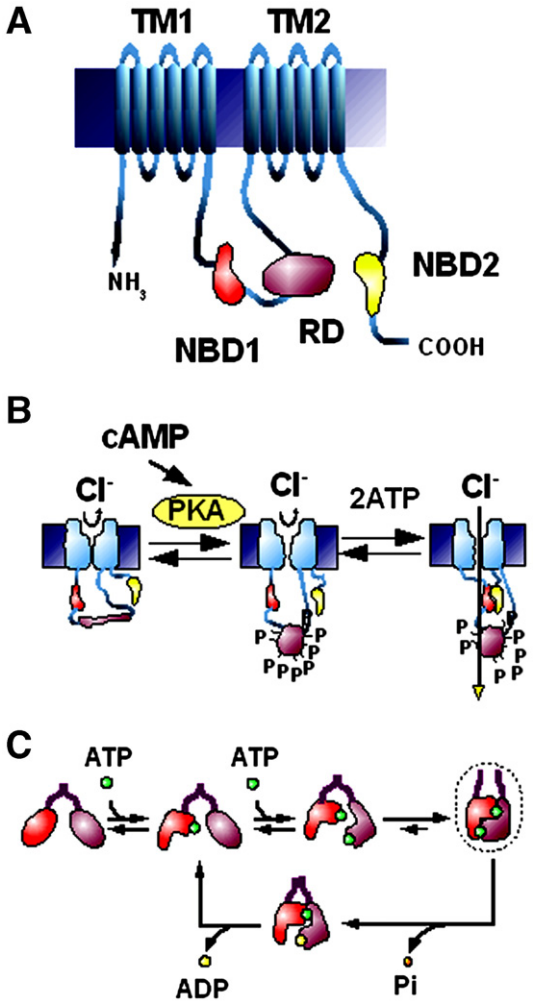

Fig. 1. A: Topology of the CFTR inserted in the cell membrane. The two transmembrane domains, TM1 and TM2, are composed by 6 membrane spanning helices each; the two nucleotide binding domains, NBD1 and NBD2, are located in the c-terminus of each TMD, in the cytoplasm, the regulatory domain, RD, joins NBD1 to TMD2. B: The functional cycle of CFTR. Protein kinase A (PKA) is activated by the cytoplasmic cAMP, and catalyses the phosphorylation of the RD of inactive CFTR, promoting a conformational transition that leads to active CFTR. The binding of ATP to the NBDs induces a second conformational change, that gates the channel to the open – chloride permeable – conformation. C: The gating cycle of the NBDs of CFTR initiates with the successive binding of two ATP molecules, that induces the dimerisation of these domains, by a quasi-irreversible transition, allowing the channel to open. The ATPase activity of the NBDs brings on the hydrolysis of one ATP, and the consequent destabilisation of the NBD1–NBD2 interaction, that causes the closure of the channel and the release of an ADP.

between the degree of loss of chloride transport and the severity of CF phenotype: the most severe loss of function mutations in CFTR are associated with the most severe forms of CF. The measurement of CFTR ability to transport chloride is considered a good, and perhaps, the only practical method to assess its activity. Hence, it is used to analyse CFTR mutants and to evaluate the effects of procedures intended to remediate the abnormal protein, such as pharmacological potentiation of defective CFTR, correction of the defective CFTR trafficking, and vectorial introduction of new proteins, or development of any other therapeutic strategy.

Physiologists and biophysicists have developed a number of different methods to quantify the transport across membranes, based on 1) the measurement of ionic currents, 2) direct evaluation of the fluxes, using chemical methods like radio-isotope tracers or fluorescent probes, and 3) indirectly, by cell membrane potential measurements. Each of these strategies has a broad number of variants that vary in their intrinsic accuracy. Therefore, the choice of the method has to be decided taking into account the biological preparation used (purified protein, isolated cells, tissue samples or whole organisms), the complexity level that it is under examination (single-channel properties, cellular responses or tissue responses), the measurable parameter (expression, level of activity, pharmacological responses), and the throughput of the experiment (large screening vs. detailed characterisation).

Frequently, scientists involved in CF research have to face the necessity to evaluate their results in terms of the functional properties of CFTR. To aid researchers in choosing the technique that best suits their needs, we have compiled a list of techniques to measure CFTR channel activity, including some comments on the systems on which they can be applied. This article does not intend to be an exhaustive description of each method, that can be better obtained from the specific references, but seeks to provide a panoramic view of the techniques as an initial resource to design an experimental strategy.

2. Activation of CFTR

As mentioned above, CFTR attains the activated state when the RD is phosphorylated by PKA (see Fig. 1B). In patch-clamp experiments and reconstituted CFTR experiments, where it is possible to perfuse the intracellular side of the membrane, phosphorylation (and the subsequent CFTR activation) can be obtained by applying the catalytic subunit of PKA.

Differently, CFTR activation in intact cells requires stimulation of the endogenous PKA by increasing the cytoplasmic concentration of cAMP. This can be obtained in an indirect mode, stimulating the cell production of cAMP via activation of adenylate cyclase by a G-protein linked hormone pathway, using glucagon, epinephrine or a beta-adrenergic agonist like isoproterenol, acetylcholine, the vasoactive intestine peptide (VIP) [5a] or adenosine [5b]. The level of intracellular cAMP obtained by hormonal stimulation and, thus, the maximal activity of CFTR, might be well below the levels obtained by using forskolin or permeant cAMP analogues.

In the laboratory, activation of adenylate cyclase is usually obtained by application of forskolin, a terpene derivative of plant origin. Extracellular application of micromolar concentrations of forskolin (0.5–10 μM) leads to an intracellular increase of cAMP sufficient to activate the PKA, with the consequent activation of the CFTR. However, high concentrations of forskolin (>20 μM) may produce unspecific responses, probably via interaction with metabolic pathways. The increase of cAMP after forskolin probably triggers a feedback mechanism, since addition of this compound to an epithelium elicits a CFTR activity characterised by an initial fast increase followed

by a reduction to a level of lower activity. This last phase is maintained as long as forskolin is present. The activity reduction could be a consequence of cAMP-concentration reduction following activation of phosphodiesterases (PDE). Therefore, to activate CFTR it is sometimes used a cocktail containing forskolin and a PDE inhibitor like IBMX [7]. However, care has to be taken when using IBMX, since it seems to have a direct action on the gating properties of CFTR [8–10], being a potential source of interference in the measurements, especially when studying the modulation or the pharmacology of CFTR.

The activation of PKA can be also obtained by the direct application of membrane permeable analogues of cAMP, like CPT-cAMP or 8-Br-cAMP. Response to exogenous cAMP seems less disturbed by feedback mechanisms. Extracellular application of micromolar concentrations of CPT-cAMP leads to a consequent activation of CFTR in a quite reproducible dose–response manner ($K_{0.5}$ ~80 μM). Phosphorylation of CFTR by PKA is a reversible reaction, as the RD is dephosphorylated by serine/threonine-specific protein phosphatases. It is thus compulsory to maintain agonists that activate PKA – or the catalytic subunit of the PKA in patch experiments – during CFTR recordings in order to sustain channel activation. Alternatively, it is possible to inhibit the phosphatases [6], increasing the effect of the PKA agonists.

Sometimes, cells or tissues in resting conditions may have intracellular levels of cAMP that are high enough to activate PKA and maintain a basal CFTR activity. It is important to take into account this possibility when studying the modulation of CFTR gating or its pharmacology, since phosphorylation can condition the channel responses [11,12]. It is, therefore, necessary to design experiments with appropriate controls of the basal condition of CFTR using specific blockers, such as $CFTR_{inh}$-172. It is important to be aware that the use of non-specific blockers, like the sulfonylurea glibenclamide may introduce further artefacts to the measurements due to its action on other transport systems, such as ATP-sensitive potassium channels [13].

The discovery that deletion of RD results in permanently activated CFTR channels has allowed to bypass the phosphorylation-dependent activation providing a useful tool for biophysical and pharmacological studies of CFTR. This deletion results in channels that are active in basal conditions but maintain most of wild-type gating properties [14,15]. However, the deletion of the RD seems not to be sufficient to completely activate CFTR channels, and further phosphorylation is

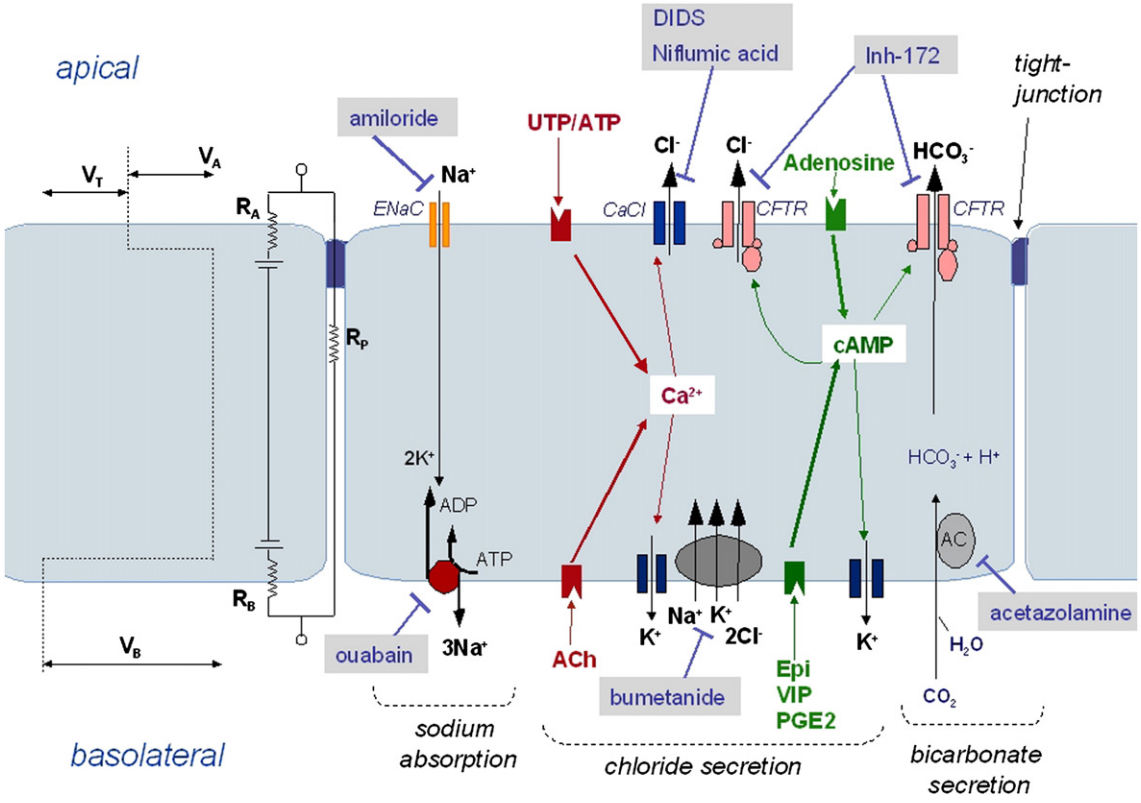

Fig. 2. Schematic representation of the most important epithelial Na^+ and Cl^- transport systems. Na^+ enters the cell from the apical side of the epithelium through *ENaC* channel and leaves the cell mainly through the *Na/K-ATPase*. Cl^- instead enters the cell from the basolateral side through the *NPCC-co-transporter* and leaves the cell at the apical side either through *CFTR* or through a Ca^{2+}-dependent channel (*CaCl*). Thus, Cl^- secretion has been divided into a *Ca^{2+}-activated system* (in red) and a *cAMP-dependent system* (in green). An increase of the intracellular Ca^{2+} concentration can be triggered by agonists acting on apical or basolateral membrane receptors, such as *P2Y* receptor activated by UTP or ATP, or the *muscarinic acetylcholine* receptor activated by acetylcholine (ACh). Similarly, a cAMP increase might be a consequence of the binding of agonists on apical or basolateral receptors, such as adenosine, beta beta-adrenergic (Epi), prostaglandin PGE2, or vasoactive intestinal peptide receptors (VIP). Also bicarbonate, formed from CO_2 by carbonic anhydrase, permeates *CFTR*. Activation of basolateral K^+ channels in a concerted way with Cl^- channels provides the driving force needed for Cl^- secretion. Grey boxes indicate some channels or transporter inhibitors.

necessary to obtain full activation. Introduction of other mutations on putative phosphorylation sites along the NBD1 may also improve the CFTR activation.

In phosphorylated channels and in the presence of ATP the two NBDs can dimerise and together form two binding sites for ATP [16,17]. This promotes the open state of the channel (see Fig. 1C). While the first site seems to be a stable binding site at which hydrolysis does not take place, or occurs at a very slow rate, at the second site ATP is promptly hydrolysed [4]. Binding of ATP at the two sites might be the power stroke that opens the channel, and the energy of hydrolysis at the second site terminates the transport cycle (Fig. 2) [16,17]. When working in conditions where the intracellular solution could be set up by the researcher (e.g. whole cell or inside-out patch-clamp configurations) it is important to work with high concentrations of intracellular MgATP (about 0.3–1 mM) to ensure that binding and hydrolysis of ATP can occur and the activity cycle of CFTR can be completed.

3. Experimental systems: cells and tissues

Experimental systems to study CFTR function span from purified protein molecules, to single cell, to artificial or native tissues (Table 1). Each system provides advantages and disadvantages. In general, the resolution and the precision of the measurements are inversely proportional to the complexity of the biological system.

3.1. Reconstituted CFTR

Purified CFTR channels can be obtained from preparations of recombinant proteins or from cell fractions and then reconstituted into planar lipid bilayers or in liposomes. The complexity of the CFTR folding limits the production of recombinant proteins to eukaryotic systems like insect cells infected by baculovirus [18] or mammalian cells [19,20]. Simpler methods, like production of proteins in bacteria are limited by the impossibility to obtain an adequate folding. Affinity chromatography-purified recombinant CFTR has been successfully used to study single-channel properties when incorporated in planar membranes [18,19]. Preparations of microsomal membrane fractions have been used extensively to reconstitute CFTR activity [21,22]. Microsomal membranes can be easily prepared from cells expressing wild type or mutant CFTR, allowing to measure the chloride transport in either planar lipid membranes or proteoliposomes, and to study the biochemistry of enriched fractions of CFTR in proteoliposomes [23].

3.2. Cells expressing CFTR

Isolated cells are the preferred system to study CFTR activity. A large number of different techniques can be applied to measure chloride transport in these preparations. Isolated cells can be obtained as primary or secondary cultures from specimens obtained from patients or animals, and grown following appropriate culture techniques. These cells have the advantage that they preserve quasi-native conditions, suitable for physiological studies. However, some care has to be taken since these cells could undergo an atypical differentiation in culture that modify their characteristics.

There is a considerable number of commercially available cell lines expressing CFTR. For example the Human Genetic Cell Repository of the National Institute of General Medical Sciences of USA, with more than 200 entries can be an interesting source of specific cell lines carrying CF mutations.

Table 1
Experimental systems for the study of CFTR chloride transport function

System	Comments on its application
Reconstituted systems	
Incorporation into lipid bilayers	Limited to detailed biophysical and pharmacological characterisation of CFTR by current measurements. Proteoliposomes can be used for patch-clamp recording, flux measurements by radiotracers and fluorescent probes assays
Proteoliposomes	
Isolated cells	
Primary cultures	Detailed biophysical and pharmacological characterisation of CFTR. Ideal for screening of expression. Can provide cellular response information. Excellent for high throughput screening design. Potential risks of incorrect membrane traffic and sorting
Cell lines expressing CFTR	
Transiently or permanently transfected cells	
cRNA expression in oocytes	Limited to biophysical and pharmacological characterisation by two electrode voltage-clamp or patch-clamp
Cell monolayers	
Made by primary cultures, cell lines expressing CFTR or permanently transfected cells	Good for biophysical and pharmacological characterisation of CFTR or for transepithelial transport — secretion or absorption. Serious difficulties for heterologous expression when the cell monolayer is formed. Can be used for long-lasting experiments. Problematic to design high throughput experiments
Biopsic samples	
Ex vivo dissociated cells	Good for pharmacology and for semi-quantitative expression tests. Adequate for transepithelial transport — secretion or absorption. Excellent to control chronic treatments on animals or patients, but difficulties for long-lasting experiments after obtaining the sample. Not suitable for high throughput experiments.
Integral epithelial samples	
In situ	
Nasal or rectal epithelia	Semi-quantitative method. Excellent to control acute or chronic treatments on animals or patients

Table 2
Methods of ionic current measurement in cells and tissues

Method	Isolated cells	Monolayers	Biopsic preparation	In situ	Comments
Patch-clamp — whole cell recording	Very good. Cell must be small (less than 50 μm diameter) to obtain reliable measurements. Excellent method for pharmacology and permeability properties. Intracellular compartment is not well controlled. Difficult to be used for gating characterisation	Not applicable	Can be efficiently used on dissociated cells. However, native cells are frequently difficult to patch	Not applicable	These techniques require expertise. Are not suitable for screening, unless automatic methods are used
Patch-clamp — single-channel recording	The best choice for gating analysis. In cell-attached configuration, integrity of the cell – and the intracellular machinery – is conserved. In inside-out configuration one has a complete control of the intracellular side of the channel (phosphorylation, ATP binding)	Not applicable	Same difficulties as above	Not applicable	As above
Two electrode voltage-clamp	Applicable to big cells as frog oocytes expressing heterologous CFTR	Not applicable	Not applicable	Not applicable	This method is susceptible to big errors if recording conditions are not optimised. Series resistance can be an important problem to measure large currents
Ussing chamber	Not applicable	Requires a high resistance cell monolayer (>500 $\Omega.cm^2$). It is the best choice to measure transepithelial currents. Can be used also to measure apical membrane currents, permeabilising the basolateral membrane	Is the best choice for measuring biopsic tissue. May be limited by the size of the sample (too small samples could introduce a big series resistance), or by the integrity of the sample	Good for epithelia in situ	Applicable exclusively to polarised cells. Big error can be introduced by the inappropriate use of electrodes (polarised or unbalanced), or by series resistances

Several academic institutions conserve and distribute cell lines generated from cystic fibrosis patients, like the Laboratory of D. C. Gruenert at the University of California in San Francisco (USA), that has a collection of immortalized bronchial epithelium cells with CFTR mutations [24].

Heterologous expression of wild type or mutant CFTR in cell lines (e.g. HEK, CHO, etc.) is also widely used. Transient expression leads to sufficient channel expression for techniques intended for measurements on single cells, like patch-clamp or imaging studies. However, transient transfection is associated to large cell to cell variability in the level of CFTR expression that may constitute a problem when performing experiments based on the measurement of a population of cells (e.g. fluorescence assays, anion fluxes, etc.). Moreover, transient transfection of the exogenous cDNA is maintained for a short time (typically 2–3 days). These problems are resolved with permanent transfections of CFTR cDNA. This method results in a clonal selection of transfected cells providing a good homogeneity of expression. In conclusion, in contraposition to the fast and simple protocol of transient transfected cells, the time consuming protocol to obtain permanent transfection has the major advantage of better quality of the results for most of the functional measurements of CFTR.

3.3. Polarised cell monolayers and epithelia

Cell monolayers constitute an excellent model of the epithelium. When cells grow in the appropriate conditions to form a polarised monolayer, the plasma membrane transport proteins distribute differentially between the apical membrane and the basolateral membrane. A strong, obvious, limitation of this system is the restriction to epithelial cells that are able to differentiate into polarised cells and form tight junctions. As the cell interstices are "sealed" by tight junctions, most of the solute transport occurs across the cell, crossing both, the apical and basolateral membranes, with mechanisms similar to those of an epithelium (see below). Therefore, to obtain reliable measurements of CFTR-mediated chloride transport across the cell monolayer, it is necessary to verify the "closure" of the interstices before attempting any measurement otherwise there is the risk of measuring leak of solutes by the paracellular pathway. This technique can be applied to some epithelial cell lines, permanently transfected cells, or primary epithelial cells. However, it is unlikely to get acceptable results using transient transfected cells, as the long time necessary to form a good enough monolayer is too long (usually between 4 and 7 days) compared to the short time with acceptable CFTR expression in isolated cells (usually less than 72 h).

An interesting variant of this technique is the electrical exclusion of the basolateral membrane by its permeabilisation by channel-forming antibiotics or bacterial toxins (see below). In this configuration it is possible to simplify the system for measuring CFTR permeation, as this protein is expressed almost exclusively in the remaining apical membrane.

Another important point that needs to be taken into consideration is that cell lines of epithelial linage do not

reproduce exactly the properties of native epithelial cell. Most of these lines are polyploids, and immortalisation surely changes the panel of expressed proteins. In fact, some of them completely lose the possibility to polarise (A549, 9HTE), others lose the apical expression of the epithelial sodium channel ENaC (CFBE14o-, 16HBE41), or may present significant differences on the protein traffic and sorting; moreover other less evident changes, such as the intracellular control of the concentration of cAMP, could have taken place. As a consequence, while any cell could be a good support to study isolated CFTR protein or the effect of compounds acting directly on the protein, studies on the regulation of CFTR or on compounds that might modify the activity of CFTR by interacting at different levels with the cell machinery, may need to be carefully evaluated. In these last cases, it could be useful to verify the effect measured on a certain cell line on other cells or, even better, on primary cultures. Different results could be also obtained measuring the effect of a given compound on CFTR from different species. Recent data [25] have evidenced for example that mice carrying the dF508 mutation have more protein in the plasma membrane. Thus, evaluation of potentiators of CFTR could be better studied there than on human cells, where the mutated protein is almost absent from the membrane, while the effect of correctors would be scarcely evaluated is this preparation.

3.4. Ex vivo and in situ preparations

Cell preparations from biopsic material are among the more physiological preparation, being an excellent choice to study the result of a given procedure on the whole organism. However, in contrast to *in vitro* preparations which are treated (immortalized) to be maintained for a long time in laboratory conditions, biopsic material in the form of isolated cells or tissue samples, could be significantly damaged during the isolation procedure. These systems have also limited time viability, before developing an adaptation to the new *in vitro* situation, moving away from the physiological conditions that justify its use, or dying. These characteristics limit the manoeuvrability of these preparations, and in some instances, require a more constringent control to asses the quality of data obtained. For example, some transport mechanisms are expressed only when the cell is polarised. A classical example is the epithelial sodium channel (ENaC), that is well expressed in the apical membrane, and is directly involved in the regulation of the apical membrane permeability in epithelia. Endogenous expression of ENaC is completely inhibited when cells are dissociated, and the rapid turnover of this channel leads to reduction of the sodium transport throught ENaC within few hours.

Similarly, in situ studies are strongly limited by the need of using non-invasive procedures to conserve the physiological attributes of the measurement, and to protect the subject in the case of measurements done in humans. It is, however, an essential method to be used to ascertain if a given mechanism described *in vitro*, or a given procedure developed in isolated systems can be adequately extended in the whole organism.

4. Methods of measurement

Methods to evaluate CFTR activity can be divided into two groups: a) electrophysiological methods, based on the measurement of i) the ionic currents or ii) the membrane potential, and b) methods to determine ion fluxes, either directly via radioisotopes or chemical methods, or via techniques that apply fluorescent probes.

4.1. Ionic current measurement

The measurement of ion currents is a method for the direct evaluation of the net ion flux across a semi-permeable barrier like a membrane (or an epithelium). The flux J (mol/s) across the barrier is described by the Fick's law as:

$$J = P\Delta c$$

where P (s^{-1}) is the permeability coefficient, and Δc is the concentration gradient (mol/l/m). For a flux of charged ions, we can convert J in electric current, I, expressed in *Amperes* (Coulomb/s), by multiplying the flux by the Faraday constant (F=96,000 Coulomb/mol). Likewise, the concentration gradient can be converted in electrochemical potential V_i for the ion i, by the Nernst's law:

$$V_1 = \frac{RT}{zF}\ln\left(\frac{c_1}{c_2}\right)$$

where R is the universal gas constant (8.314 V Coulomb K^{-1} mol^{-1}), T is the absolute temperature (K), z the valence of the ion, and c_1 and c_2 are the concentrations at either side of the barrier.

The measurement of ionic currents can be obtained via voltage-clamp. A voltage-clamp amplifier is a device that allows to maintain a fixed command potential, V_{com}, between the two sides of the barrier, while the circuit measures the current necessary to maintain this potential, that is equal (and opposite) to the current that flows across the membrane. Hence, for a system with an electrochemical potential V_i, at a given command potential V_{com}, the current is described by the Ohm's law as:

$$I = G(C_{com} - V_i)$$

where the conductance, G (Siemens), is the inverse of the resistance. There are several different types of voltage-clamp methods, suitable for different preparations, from small patches of membranes to whole cells, very big cells, or to measure currents across complete epithelia. An important characteristic of all voltage-clamp techniques is their considerable time resolution, providing information on the kinetic properties of the CFTR channels and on the precise time course of a modulatory or pharmacological intervention. A briefing on the most common voltage-clamp methods applied to CFTR functional measurements are described in Table 2.

4.1.1. Patch-clamp

The patch-clamp technique is an electrophysiological technique originally designed to measure single-channel events [26], and successfully extended to the measure of whole cell

Table 3
Methods to measure the membrane potential in cells and tissues

Method	Isolated cells	Monolayers	Biopsic preparation	In situ	Comments
Cell membrane potential	Fair	Fair	Fair	Difficult	Requires expertise. Not suitable for screening
Potential sensitive fluorescent dyes	Good	Good, but difficult to interpret	Good	Difficulties loading the probe to cells and accessing the tissue with optical instrumentation	Good qualitative method. Good for high throughput screening.
Transepithelial potential	Not applicable	Good	Fair	Most suitable method for non-invasive measurements in live animals and human patients	Good technique for in situ. In isolated epithelia, it is a good choice for inexperienced operators

currents in small cells [27–29]. The technique is based on the electrical isolation of a small patch of membrane by means of the tip of a glass micropipette. According to the size of the isolated patch of membrane and on the density of CFTR expression in the membrane, a single channel or multiple channels can be recorded. The patch-clamp technique has been successfully applied to almost any type of dissociated cell, as far as their surface is accessible and clean. Cell lines usually are easier to patch than primary cultures and freshly dissociated cells. Several details on the patch-clamp preparation condition the success of patching, like the temperature, presence of divalent cations, and presence of proteins or nucleotide in the pipette. Unfortunately, there is no fixed recipe to obtain successful patches, but ideal conditions have to be found for each preparation.

Several different variants of the original technique were obtained. In the cell-attached configuration the integrity of the cell is maintained and currents are measured upon stimulation by membrane permeable agonists (see above). However, this configuration has the disadvantage to have no access to the intracellular side of the channel, and a very difficult access to the extracellular side. In addition, the cell resting potential is unknown. There are two excised patch configurations, the inside-out and the outside-out configuration. The inside-out configuration is probably mostly used to record single CFTR channels. With this configuration, the intracellular side of CFTR is exposed to the bath solution, and therefore can be easily perfused with various solutions containing kinases, nucleotides and other substances. In this configuration, it is possible to induce the phosphorylation of CFTR by direct application of PKA catalytic fraction, and to keep it in the solution all along the experiment to keep CFTR in the activated state.

The whole cell configuration is also extensively used to record CFTR chloride currents, in particular to study pharmacology and channel biophysical properties. In this case, because the cell is dialysed by the pipette solution, it is necessary to supply the cell with PKA and ATP directly in the pipette solution. A variant of the whole cell configuration is the perforated patch. In this configuration, the patch of membrane under the pipette is “perforated” by channel-forming antibiotics (as nystatin, gramicidin) or bacterial toxins (alpha-toxin), gaining electrical access to the cytoplasmic side, but avoiding the lost of intracellular macromolecules, keeping most of the cell machinery. This is a very good method to study the modulation of channels that strongly depends on the intracellular milieu.

The patch-clamp technique is among the highest resolution methods to study ionic currents. In the appropriate conditions, it is possible to resolve single-channel events as small as 0.1 pA, with a bandwidth of 5 kHz. It is indeed the best choice to study the molecular mechanisms of the gating of CFTR and its modulation. The traditional, hand-operated method is not appropriate for screening experiments, because it requires some expertise and manual ability, and the acquisition of data could be extremely time consuming, resulting in a low-throughput system. A good alternative for screening is the use of automated patch-clamp systems that have been recently introduced in the market [30–32].

4.1.2. Two electrode voltage-clamp

Two electrode voltage-clamp is a good method to record CFTR currents expressed in frog oocytes [33]. This technique is relatively easy and can be appropriate for the screening of substances in a moderate throughput. However, when using two electrode voltage-clamp, one has to be conscious about the resolution limitations due to the big size of the oocytes (about 1 mm diameter), that generates problems with correct control of the potential, especially when relatively high currents are recorded [34]. This problem can be avoided applying the patch-clamp techniques to oocytes, improving the time resolution, but reducing the throughput of data.

4.1.3. Ussing chamber

The Ussing chamber, originally introduced by Prof. Hans Ussing to study the ionic transport across the frog skin [35], can be applied to study ion transport through various types of epithelia and cell monolayers. The method consists of the measurement of current (or voltage) between two hemi-chambers separated by a section of an epithelium or an epithelial cell monolayer grown on a permeable support. Fig. 2 shows a scheme of an epithelium (or cell monolayer).

The distribution of channels and transporters may vary considerably between epithelia of different organs, but most surface epithelia, including the airways epithelia, are basically sodium absorptive. In fact, the proximal airways have the important role of removing water produced deeply in the large surface of small bronchi branches and alveoli. In absorptive epithelia sodium enters the cell from the apical side through the

epithelial Na channel ENaC and leaves the cell at the basolateral side through the Na/K-ATPase. Sodium movement is accompanied by chloride, passing probably through the paracellular pathway, and osmotic water. Given the intracellular and extracellular concentrations of chloride, there is no driving force for chloride movement in resting conditions. However, it is believed that, under hormonal stimulation, activation of basolateral potassium channels causes cell hyperpolarisation providing the driving force that permits chloride secretion. Chloride enters the cell basolaterally through the sodium, potassium and chloride co-transporter (NKCC), and leaves the cell at the apical side through chloride channels such as CFTR.

The electrical equivalent circuit of an epithelium (see Fig. 2) consists of two resistors, R_A and R_B, representing the apical membrane and the basolateral membrane respectively, in series with two batteries that yield the potential difference of these two membranes, V_A and V_B. The transepithelial potential difference is defined as $V_T = V_B - V_A$. In parallel there is another resistance, R_p, called also "leak resistance", representing the cell interstice where ions could permeate, named the paracellular pathway. The total resistance of the epithelia, R_T, is therefore described by:

$$R_T = \frac{R_p(R_A + R_B)}{R_p + R_A + R_B}.$$

Leakiness of the tight junction between cells, that determine R_p, represents an important contribution to R_T. To measure the current flowing through the channels, that is the transcellular current, it is necessary to have a reduced flux by the paracellular pathway, i.e. to have high R_p values. Hence, to obtain good measurements of the currents across channels, it is necessary to have epithelia with relatively high R_T values. According to the value of the transepithelial resistance, epithelia have been arbitrarily classified as "tight", with $R_T \geq 1.5$ KΩ cm^{-2}, and "leaky", for $R_T < 1.5$ KΩ cm^{-2}. Leaky epithelia have higher water and sodium permeability than tight epithelia, but cannot maintain or create a gradient. Leaky epithelia have $V_T < 10$ mV, while higher V_T values, up to several tens of mV can be observed in tight epithelia.

The classical parameter measured in an epithelium mounted in an Ussing chamber is the "short-circuit current", I_{SC}, that is the current across the epithelium when is short circuited, i.e. V_T is clamped to 0 mV. As mentioned before, in most *in vivo* epithelia and in epithelia that keep some of their physiological features, sodium contributes to a significant extent to I_{SC}, and therefore, to evaluate the chloride current flowing through CFTR channels it is necessary to inhibit this cation current. The transcellular sodium current can be completely eliminated by the addition of micromolar concentrations of the ENaC blocker amiloride to the apical side of the epithelium. Attention has to be paid to the dosage of amiloride as at high concentrations it could modify also other mechanisms that might be present in some epithelia. Assuming that the paracellular current is small enough, after application of amiloride, the transepithelial current approximates zero. In this condition, it is possible to activate chloride fluxes through the different chloride channels present in the epithelial, including CFTR. As mentioned before, addition of forskolin or permeable cAMP analogues activates CFTR. However, to obtain another indication that the current measured is indeed conducted by CFTR, it is desirable to use specific blockers at concentrations as low as possible. On most human cells, $CFTR_{inh}$-172 blocks CFTR completely at concentrations between 5 and 10 μM. In addition, appropriate controls to estimate the paracellular contribution to the total current are necessary to obtain reliable data.

Several variations of the classic Ussing chamber have been applied to a large number of different epithelial preparations to measure CFTR activity, from monolayers formed by cells grown on permeable supports developed to be inserted in a chamber [12,36], to biopsic samples [37–40]. An interesting variation of the method is obtained by permeabilising the basolateral membrane with an antibiotic, like nystatin or amphothericin [41], or by *Staphylococcus aureus* alpha-toxin [42]. These agents form pores in the membrane, that becomes a low resistance pathway for ions across the basolateral membrane virtually suppressing R_B. It results in a configuration where (assuming a high paracellular resistance) most of the current flows across the apical membrane, improving considerably the specificity of the measurement of CFTR channels. Note that channels formed by antibiotics or alpha-toxin are permeable to ions, but not to macromolecules, or even low molecular weight substances, like nucleotides, leaving the intracellular composition, except for ions, quite constant.

4.2. Membrane potential measurements

The cell membrane potential is firmly coupled to the membrane permeability and the ionic gradient across the membrane. The resting cell potential, V_r, can be estimated from the Goldman–Hodgkin–Katz equation as:

$$V_r = -\frac{RT}{F}\ln\left(\frac{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_O}{P_K[K]_O + P_{Na}[Na]_O + P_{Cl}[Cl]_i}\right)$$

where P_j is the permeability of membrane for the ion j, and i and o represent the intracellular and extracellular concentrations of each ion, respectively. A modification of the membrane permeability for any ion, for example as a result of activation or inhibition of a channel, will result in a modification of the membrane potential. However, quantification of such modifications in terms of change of the conductance of a channel is not immediate. Modification of the membrane potential will influence other channels, in particular several types of inward rectifier and voltage dependent potassium channels and chloride channels of the CLC family, which, in turn, will modify the membrane potential. When the channel under investigation is over-expressed in the cell, accumulation or depletion of the permeant ion will create a further inconvenience, as the ion electrochemical gradient will influence the potential. In conclusion, while membrane potential measurements can be useful for a simple control of a modification of CFTR (or any other) channel function, quantitative analysis of such modifications will be difficult. It is important to notice that in physiological

conditions, i.e. when cells are surrounded by a high extracellular chloride concentration, the chloride equilibrium potential is very near to the membrane potential. Thus, modifying the chloride activity, either activating or inhibiting CFTR, does not produce a significant change in the membrane potential. Therefore, to detect the CFTR activity by measuring the membrane potential it is necessary to reduce the extracellular chloride to obtain an electrochemical gradient that produces a voltage change when the chloride permeability is modified.

Direct methods for measuring the membrane potential are the use of intracellular microelectrodes and the whole cell patch-clamp recording, in the current-clamp configuration (Table 3). In fact, these are the appropriate techniques to control the validity of other methods, like those that use fluorescent voltage sensitive dyes [43]. Intracellular microelectrodes have several advantages over the patch-clamp. Patch-clamp requires the direct access of the pipette to the membrane, that in intact epithelia is often possible only after enzymatic treatments that may change the state of the tissue. On the other hand, the fine tip of the electrode prevents damage and preserves the physiological micro-environment of the cell, while the patch-clamp electrode dialyses the cell cytoplasm. These two techniques can be used for almost any preparation, within the limits of accessibility of cells and membranes, but require expertise and manual ability. Complexity of the method (mechanical stability, electrical screening, micro-manipulation of the electrodes under microscope observation) makes it difficult to apply on in situ preparations, or to use in high throughput experiments.

An alternative approach to measure the membrane potential is to use fluorescent dyes [44]. The bisoxonol compound DiSBAC2(3) has been used to assess the expression of CFTR [45]. A precise calibration of the fluorescent signal against the absolute cell membrane potential is hardly possible, but the DiSBAC2(3) signal is very well correlated with the potential changes [43]. This compound presents several advantages including a rapid and simple handling, the possibility to apply the method to a large number of airway cells, for example cells obtained from nasal or bronchial brushes, and it is suitable for high throughput screening experiments. However, the time course of DiSBAC2(3) fluorescence response to a change of membrane potential has a time constant of hundreds of seconds [45,43], thus precluding the implementation of kinetic experiments. Kinetic properties of the membrane potential changes are better assessed with electrophysiological methods, that have time responses between tens of microsecond to few milliseconds.

As mentioned above, when polarised cells adhere to each other by tight junctions, forming an epithelium, a transepithelial potential, V_T, is mainly a result of the contribution of the sodium and chloride transport systems (see Fig. 2). Thus, measurements of changes of transepithelial potential V_T in response to channels activators or blockers, or changing the permeant ions concentration, could reveal the contribution of different channels to the transepithelial potential. This technique is particularly suitable for studies on whole animals and can also be applied to human patients [46–50]. Transepithelial nasal or rectal potential can be measured introducing an electrode in the liquid layer adjacent to the apical side of the epithelia (the nose or the rectal cavities), and measuring the potential difference against a hypodermic reference electrode. Several commercial devices have been designed to normalise the measurements, and to allow perfusion of the epithelia with solutions of different composition. The sequential additions of amiloride to block ENaC currents, low chloride solution to increase the driving force for chloride secretion, cAMP to activate CFTR, and a CFTR inhibitor, provide a good insight in the level of CFTR activity. The method has been proved to be appropriate for diagnosis or to evaluate the result of clinical tests [51,52].

Transepithelial potential is also a good parameter to evaluate the quality of polarised cell monolayers in culture, as many tight monolayers develop a transepithelial potential. We consider that this method could be used as a simple technique to evaluate CFTR activity. Potential difference can be simply measured between two Ag||AgCl electrodes, using a voltmeter. Electrodes can be made with a silver wire that has been immersed for a couple of minutes into bleaching. To avoid electrode unbalance when using low chloride solutions, and to assure the sterility of cells, the electrodes are connected to the chamber solutions through single-use Ringer-agar bridges. Modern digital multimeters are ideal for these measurements, as they have adequate impedance, and can be also used to measure the transepithelial resistance by switching the instrument to the ohmmeter mode. The easy access to the monolayer solutions allows the application of modulators and drugs similar to those described above. A further advantage is that, if experiments are done with adequate precautions to avoid contamination, measurements can be repeated in time, allowing the design of chronic experiments. This is probably the easiest and cheapest method to evaluate CFTR function.

4.3. Chloride fluxes assays

Chloride fluxes can be determined by evaluating the mass of ions incorporated into stimulated cells, or the release of ions from the stimulated cells. The use of radioactive tracers is probably one of the most precise methods to determine the ionic flux. It has the advantage that even in the absence of a net flux of chloride, when the electrochemical potential is zero, it is possible to determine the activity of CFTR from the unidirectional radiotracer movement. The method is quite well applicable to most preparations, except the in situ systems, due to the difficulty in using radioactive material on living animals or human patients (Table 4).

An equivalent method is based on measurements of iodide flux. Because the CFTR channel is permeable to iodide, it is possible to determine the efflux of this ion from previously loaded cells by a colorimetric assay [53] or using an iodide-selective electrode [54–56]. All these flux measurements have the inconvenience that, in most cases, efflux determination is more reliable than influx measurement, but implies that cells have to be preloaded with the radioactive marker or iodide, by relatively long incubation.

Fluorescent probes can be used to estimate the intracellular chloride (or iodide) concentration changes, with a reasonable time resolution. There are several different fluorescent probes to apply for halide determination, as MQAE (*N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide) [57,58], SPQ (6-methoxy-*N*-(3-sulfopropyl) quinolinium) [59,60,57], MEQ (6-methoxy-*N*-ethyl-1,2-dihydroquinoline) [61,62]. All these dyes have to be loaded into cells before performing the experiment.

Table 4
Methods to measure the chloride flux in cells and tissues

Method	Isolated cells	Monolayers	Biopsic preparation	In situ	Comments
Radioactive tracers	Excellent method. The most reliable to evaluate cell permeability	Can be used to measure transepithelial transport. Multi-compartmental analysis required	Applicable to epithelial samples or to dissociated cells	Several difficulties for the use of radioactive material on live animals. Not suitable for patients	Is about the best method to measure fluxes. Can measure ^{36}Cl, ^{125}I. Formal complications can arise for the use of radioactive tracers in some ambients.
Iodide electrodes	Very good for efflux of iodide preloaded cells. Not good for iodide influx	Not suitable for transepithelial flux	Applicable to dissociated cells, if they could be loaded with iodide	Not applicable	Iodide electrodes can be unstable. Iodide sensitive resin has to be maintained in optimal conditions to have reproducible results. Repetitive calibrations are necessary. Both, iodide and the electrodes are light sensitive
Iodide colorimetric methods	Can be used for the influx, but best results are obtained with iodide efflux	Not suitable for transepithelial flux	Applicable to dissociated cells, if they can be loaded with iodide.	Not applicable	Range of measurement is limited. Too small fluxes or to high iodide accumulation will not be detected properly.
Chloride probes	Excellent	Very good	Excellent	Difficulties loading the probe to cells and accessing the tissue with optical instrumentation	Use chloride sensitive dyes. Quenching by iodide could be used for fast response measurements
Green fluorescent protein based assay	Excellent	Very good	Not suitable	Not suitable	Good method to measure fast responses by iodide quenching. Require transfection of the probe, rendering unsuitable for biopsic or in situ preparations.

Alternatively, it is possible to transfect cells with green fluorescent protein variants, like some YFP mutants, whose fluorescence can be differentially quenched by various halides [63–65]. While GFP-CFTR transgenes have been successfully used to express different CFTR proteins and track their trafficking towards the plasma membrane [66–68], the link of GFP or other flags to CFTR might disrupt the interactions with PDZ motifs (flags located near the C-term of CFTR), the regulation by syntaxins (flags linked near the N-term), or with other regulatory factors. Therefore, care must be taken with generalisation of results regarding the regulation of the protein when these results are obtained using tagged-CFTR, since the experimental settings might not reflect physiological conditions. Conversely, co-expression of CFTR with other fluorescent proteins, as halide-sensitive YFP seems to not affect the CFTR physiology.

Different measurement modalities can be applied to detect chloride transport activity. Upon activation of CFTR channels, changes in the intracellular chloride can be detected directly with a chloride-dependent fluorescent dye, or indirectly as the dye fluorescence quenched by other permeable anions, like iodide or bromide. When measuring fluxes using chloride-dependent fluorescent probes, a chloride gradient must be imposed. Fluorescent responses can hardly be calibrated to obtain absolute flux values, but changes in fluorescence should be related to the chloride channel activity. The flux is usually estimated in terms of the maximum derivative of the fluorescence signal [65]. When using fluorescent probes, it is important to take into account the possibility of signal interferences by intracellular changes on pH, interferences caused by pharmacological substances assayed, or the autofluorescence of some of such substances, that can alter the response of some fluorescent dyes.

Fluorescent dyes have the advantage that they can be applied to almost any preparation, while the use of YFP on biopsic material is limited by the necessity to transfect cells with high efficiency. However, when a stable YFP-transfected cell line is obtained, it can be used to measure the functional expression of CFTR without the preliminary steps of dye loading, and without the decay of signal with time due to leakage of dye. These techniques, in particular using the quenching of a YFP mutant by iodide, have been successfully applied to high throughput screening techniques [63,64].

5. Concluding remarks

We have presented a list of methods to evaluate the functional properties of CFTR as the chloride transport across the CFTR channel. These methods provide different degrees of precision of the measurement, from absolute values of ionic flux, to semi-quantitative evaluation of activity, to a simple qualitative assess of CFTR expression. On the other hand, every technique is suited to a specific preparation. Therefore, the choice of the method to be used will depend on three main factors:

1- What is the preparation to be used? Methods to evaluate CFTR function in biopsic samples of epithelia may not be appropriate for isolated cells.

2- What is the objective of the measurement? The experimental design to measure the molecular mechanism of CFTR, or a detailed pharmacological analysis is different than the design to assess qualitatively but rapidly the expression of CFTR or a high throughput screening of a chemical library in drug discovery programs. In general, there is an inverse relation between the precision of the technique and the throughput of measurements.
3- What is the experience of the operator? All methods require special training to obtain reliable results and to interpret them. Thus, time spent in training on a given technique will avoid fake results or mis-interpreted data.

It is noteworthy that, when measuring ion fluxes mediated by CFTR, chloride and other ions could also flow through other channels, pumps and transporters, interfering with the evaluation of CFTR activity. Therefore, it is necessary to design adequate controls, using inhibitors of specific ion pathways or by modifying the solutions composition in order to change the ion gradients, and thereby dissecting carefully the CFTR contribution to the ion flux.

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